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AB Intraperitoneal ***insulin*** infusion of ***Genapol*** stabilized ***insulin*** via implantable devices significantly improves diabetes

High Antigenicity of Intraperitoneal Insulin Infusion via Implantable Devices: Preliminary Rat Studies

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Intraperitoneal insulin infusion of Genapol° stabilized insulin *via* implantable devices significantly improves diabetes control and hypoglycemia frequency in type1 diabetes while it increases insulin antibody levels. Causes for this particular antigenicity remain unknown. The role of insulin modifications occurring in the reservoir on the antigenicity observed was assessed by comparing the antigenicities of the insulin coming from the vial or from the pump reservoir. Rats were injected intraperitoneally with insulin sampled either from a vial (group 1) or from a pump reservoir during a refill of a clinical trial (group 2). Two control groups, one without insulin, the second one receiving a mixture of silicone and insulin were also studied. Human insulin antibody levels were assessed by RIA 10 days after 4 weekly immunizations. AIA levels were higher in group 1 compared to group 2 ($P = 0.003$ for the first experiment, $P = 0.04$ in the second experiment). The increased antigenicity of the insulin sampled from the implanted pump might be due to the insulin modifications occurring during the storage in the device. Insulin aggregates could be involved in this antigenicity since they are known to be antigenic and their concentration was shown to be related to the amplitude of the antigenic response.

■ Key words: - Insulin Antibodies – Insulin Stability – Insulin Aggregates – Type 1 Diabetes Mellitus- Insulin Therapy

Intraperitoneal insulin (IP) absorption has been shown to be rapid and reproducible. It is more physiological than intravenous or subcutaneous administration since 99% of the insulin administered intraperitoneally is absorbed *via* the portal route and directly reaches the liver improving hepatic protein synthesis and lipid profile [1].

Long-term IP insulin infusion requires the use of implantable devices to avoid infectious complications [1]. Clinical trials have shown the feasibility of this mode of therapy as well as its efficacy in allowing good diabetic control and in avoiding severe hypoglycemia [2].

IP insulin therapy *via* implantable devices has been shown to dramatically increase ($p < 0.001$) on a long-term basis (> 3 years) insulin IgG antibody levels (AIA) in about 40% of type 1 diabetic patients [3,4,5]. In these patients significantly ($P < 0.05$) higher post-prandial glycemia was observed. Some rare cases of clinically relevant insulin resistance or on the contrary important glycemic drops during night-time were reported [3,5]. Long-term complications remain to be determined.

The Hoechst 21PH insulin used in these clinical trials, being a humanized, neutral, regular insulin should have a low antigenicity.

Causes for the high antigenicity observed with this mode of therapy, are still unknown [6].

Different factors may be involved in this high antigenicity:
- The intraperitoneal route of administration is specifically used to produce antibodies in mice [7, 8].
- Hoechst 21PH is stabilized with a surfactant; the Genapol° (polyethylene – polypropylene – glycol), which may act as a carrier and boost antibody production.
- Insulin modifications or degradation products specifically induced by insulin storage in the pump reservoir could also be responsible for this increased AIA formation.

Insulin physical stability has always been one of the main issues concerning the implantable pump technique [9]. All conditions in implantable devices are found to favour the aggregation process such as thermal exposure, long-term contact with metallic and synthetic surfaces, mechanical stresses in the pump itself, and agitation are known to induce fibrillation of insulin in neutral solution [9]. Since the finalization of the Hoechst 21PH insulin, the problem seemed to be solved and large-scale clinical trials began in 1989. In 1992 uncontrolled decreases in pump insulin rates were reported, this incident frequency increasing over time. Insulin aggregates were observed in the system pulling the insulin out of the Minimed 2001 reservoir as well as in insulin sampled. Further inquiries demonstrated that the decrease in insulin stability was due to minor changes in insulin preparation and decided to fulfill new requirements of European Pharmacopoeia [10,11].

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Aim f the Study

In order to better understand the causes of the high antigenicity of the insulin delivered *via* an implantable devices, we tested the antigenicity of the insulin sampled from a device reservoir during 6 weeks in a clinical trial compared to native insulin sampled directly from a vial.

Material and Methods

Wistar rats weighing approximately 230 g at the beginning of the study were tested. Four groups of 5 females (Centre d'Elevage Depre, St Doulchard, France) and 3 groups of 10 males (IFFACREDO, L'arbresle, France) were studied. In clinical observations, Hoechst21PH antigenicity was high in both male and female, therefore we wanted to test both male and female rats.

The insulin studied was the one used in the clinical trials: the 21PH human, semi-synthetic Genapol° stabilized insulin (Hoechst A.G. Frankfurt, Germany) at a concentration of 400 U/ml. The Genapol° at a concentration of 10 µg/ml acts as a surfactant avoiding the deformation of insulin molecules, which may lead to insulin degradation (loss of biological activity and fibrillar aggregates formation) [12].

In vitro stability of the insulin used was assessed on turbidity (Abs 450 nm) and aggregates concentration results determined by Dynamic Light Scattering [13]. Aggregates concentrations could not be assessed in the particular samples used in the rat study, but aggregates concentrations from other samples of the same batch were obtained in conditions considered to be close to clinical conditions in the expert report (5 pumps shaken at 37 °C during 60 days and 90 days, standard deviations vary from 15% to 65% of the means) (data from B. Van Antwerp, Minimed company collected for the "Expert Clinical Report" [Pr Pinget June 1999] in order to get the Ministry of Health approval for clinical usage). Aggregates concentrations were 0 U/ml in the control vial and 20.6 U/ml after a 60 days storage in the pump reservoir sample (41.2 U/ml after 90 days). Turbidity result for this batch was > 2.2 after 90 days.

Silicone (Rhodorsyl 43V120F*) was used as a medical oil for emulsioning the vial insulin solution at a volume of 1/1 to test the maximal ability of the rats to respond to human insulin (maximal antigenic response to insulin).

The insulin used in the rat study was sampled from a Minimed MIP 2001 (Minimed Technologies, Sylmar, CA, USA) during a routine refill in our department. Pump reservoir was emptied from remaining insulin and refilled with fresh insulin after 6 weeks of storage in the pump. Pumps and clinical trial design have been described elsewhere [14].

Rat serum was tested for human insulin antibodies using the "Sanofi Diagnostics Pasteur" kit. Anti-insulin antibodies were determined by a radioimmunoprecipitation assay in liquid phase. Serum containing AIA was incubated for 24 hours with human A14 iodine 125-labelled insulin. The iodine 125-insulin bound to anti-insulin antibody fraction was separated from the free iodine 125-insulin fraction by precipitation with polyethylene glycol. After centrifugation, radioactivity counting of the pellets (bound fraction) allowed calculations of the binding percentage of labeled insulin. Binding percentage of a negative control serum was subtracted from each result and 2.5% fixed as positive threshold for humans.

Insulin antibody levels induced using Hoechst 21PH insulin sampled directly from the vial were compared to AIA levels induced by Hoechst 21PH remaining insulin sampled from a pump reservoir during a routine refill in a clinical trial.

Study Design and Protocol

Two experiments were conducted. In the first experiment 2 groups of 5 female Wistar rats were injected intraperitoneally with 200 µl of insulin sampled either directly from the insulin vial (group 1) or from a pump reservoir (group 2). One hundred microliters of silicone mixed with 100 µl of Hoechst insulin were injected intraperitoneally to a group of 5 female Wistar rats. Food and honey were left in cages *ad libitum* in order for the rats to counteract insulin action and avoid hypoglycemia.

Four immunizations were performed every 10 days. A control group of 5 female rats did not receive any insulin. Seven hundred microliters of blood were sampled by tail bleeding under general anesthesia using Hypnovel° (Roche, Neuilly/Seine, France) administered intraperitoneally (30 mg/kg). Insulin antibody levels were assessed on plasma in the control group and 10 days after the last immunization in the 3 other groups.

In the second experiment 2 groups of 10 male rats were immunized using exactly the same protocol as used in the first experiment with respectively 200 µl of insulin sampled either directly from the insulin vial (group 1) or from a pump reservoir (group 2).

After logarithmic transformation of the data, Mann-Whitney test was used to compare the mean AIA levels evolution between the 2 insulin treated groups in the first experiment. After verification of the normality (Levene test for variability) of the population of the second experiment, a Student's *t*-test was used. Because of the heterogeneity of the results within the groups, the highest and lowest values were excluded in each group. Statistical significance was defined as $P < 0.05$. Statistical testing was performed using the "BMDP" statistical software (Inc. Los Angeles, USA).

Results

Mean AIA levels obtained in each group in both experiments are summarized in Tables **1** and **2** and Fig. **1a** and **b**. In the first experiment mean AIA levels were of 0.0% ± 0.0% in the control group. After immunization, mean AIA levels were 21.7% (6–57.6%) in the silicone insulin group (n = 5). Mean AIA levels were significantly lower in group 1 (n = 5), 1.66% (1.4–2%) than in group 2 (n = 4), 9.75% (5.2–21.5%) ($P = 0.003$). One rat died from an abdominal wound after the second immunization in group 2.

During the second experiment mean AIA levels reaching 0.52% (0–1.1%) in group 1 (n = 8) were lower ($P = 0.04$) than in group 2 (n = 8) reaching 0.96% (0.4–1.9%).

Table 1 AIA levels (%) in the two experiments
First experiment

Controls	Silicone	Group 1	Group 2
0	7.9	2	21.5
0	19	1.6	7.1
0	57.6	1.4	5.2
0	6	1.8	5.2
0	18	1.5	

Table 2 AIA levels (%) in the two experiments
Second experiment

Group 1	Group 2
0.8	0.8
1.1	1
0.2	1.1
0.6	1.1
0	0.4
0.5	0.6
0.7	0.8
0.3	1.9

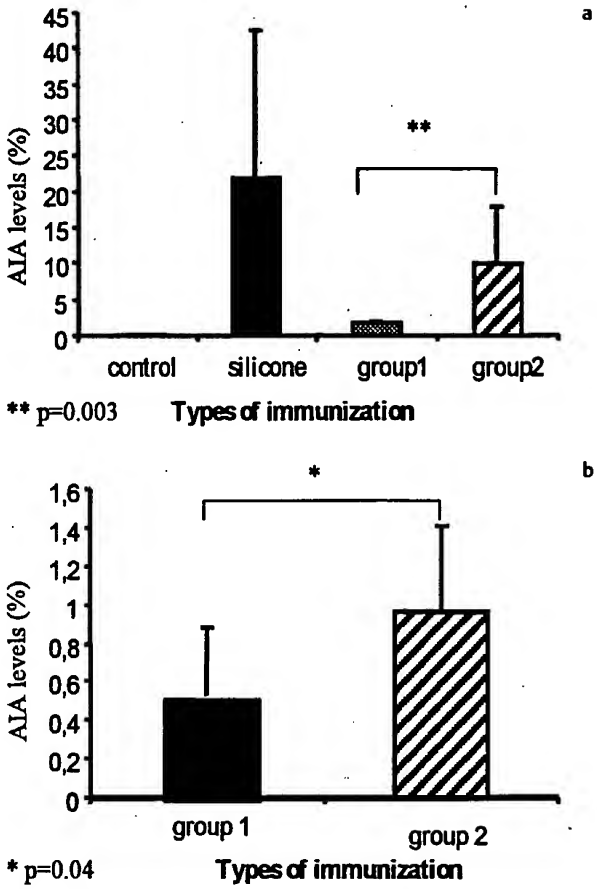

Fig. 1 Mean (± SD) AIA levels observed in both experiments. **a** First experiment results. **b** Second experiments results.

Discussion

In this study Hoechst 21PH insulin sampled from an implantable pump reservoir during a refill induced higher AIA levels than insulin directly sampled from a vial. These results exclude the Genapol° or the IP route of administration as responsible factors for the high antigenicity of this mode of therapy. Insulin composition and route of administration being comparable for each group, some insulin modifications occurring in the pump reservoir are likely responsible for the increased antigenicity observed. The insulin sampled from the reservoir was the one infused to the patients. These results are comparable to the high antigenicity observed during the clinical trials.

During the clinical studies performed prior to the modifications of insulin production [15], the main modifications observed during insulin storage in the pump reservoir were essentially insulin degradation into desamido-insulin and high-molecular-weight derivatives. After the insulin modification of production, fibrillar aggregates linked to physical degradation were found in the pump reservoir and ejection system, their accumulation causing a pump rate slow-down in clinical trials [10].

Desamido-insulin does not seem to be highly antigenic in rabbits compared to native insulin [16]. Fibrillar insulin aggregates have been shown to be more antigenic because of the formation of new epitopes [17]. A poorly soluble polymer is likely to be engulfed by macrophages, which is a potent stimulus to antigenicity [18]. A protein chemically or physically degraded will lead to an increased immune response by IgG antibodies [1], the AAI observed in the clinical and rat trials are IgG. In rabbit immunization experiments partially fibrillated insulin samples exhibited a tendency to increasing formation of IgG antibodies with increasing contents of fibrillar insulin when compared with a reference sample of native unfibrillated insulin [19].

Some authors have found [19] a relation between the aggregates concentration and the antigenicity.

A clinical preliminary study [20] showed that when using Hoechst 21PH via an external pump, AAI levels remained stable. The lack of antigenic response could be due either to the use of the subcutaneous route of administration or to the low duration of insulin storage (3 days) in better conditions of temperature. The proportion of degraded insulin would thus be lower in external devices compared to implantable devices. The rat data would confirm the second hypothesis since a difference in antigenicity potency is observed using the IP route of administration, insulin storage and eventual modifications being the only difference between the 2 groups.

Involvement of other insulin modifications occurring in the implantable pump reservoir cannot be ruled out in the high antigenicity observed, in particular, silicone oil. Fibrillar aggregates have been found in the insulin sampled from pumps reservoir. Concentrations ranged from 0.3 to 1.4 µg/ml (mean

0.65 µg/ml) in about 89 pumps explanted during the clinical trial (data kindly provided by B. Van Antwerp, Minimed Company, Sylmar, CA). Silicone oil is commonly used to lubricate the syringes used to refill the pump reservoir, and probably accumulates. Silicone has been reported to be an immunologic adjuvant when mixed with a foreign protein. [21]. The use of silicone at a concentration of 1/1 in our silicone group confirms its potency as an adjuvant for insulin immunogenicity in the conditions of experiment. The adjuvant potency of silicone at concentrations close to the ones found in the clinical studies (0.65 µg/ml) is improbable but is yet to be determined.

As always in animal studies, it is necessary to be cautious before extrapolating to humans. The difference between the AIA levels obtained in the first and second experiment may eventually be explained by the difference of gender of the rats studied. Females have usually higher antigenic response than males [22, 23]. The low amplitude of the antigenic response in the male group may be a limitation in our study since the levels are below the specific binding threshold, but it is important to note that all controls had AIA levels of 0%. Besides, even if the increment after immunization is hard to be defined as significant, what we wanted to assess here was the potential for the Hoechst 21PH to induce antibody formation using either fresh insulin or stored in the pump insulin. The difference of AIA level induced between these 2 groups is significant. A Freund's adjuvant could have been used in order to boost the antigenic response and increase the amplitude of the AIA levels, but we thought that using the pure antigen without any adjuvant seemed to be more physiologically sound [19].

As in clinical trials, antibody levels are very different within subjects in the same group, this is also commonly observed and is due to the fact that the amplitude of antigenic response is controlled by the genes of the major histocompatibility complex in rats as in humans [23, 24, 25, 26].

In human studies, type 1 diabetic patients tend to form more AAI than type 2 or simple gestational diabetic patients who react differently [24], this being probably due secondarily to their particular MHC system. The rats we studied were non-diabetic. The use of autoimmune diabetes-prone rats such as the BB rats would perhaps have enhanced the antigenic response as well as the specificity or homogeneity of this response.

In our study the antigenic response observed is heterologous, as opposed to the homologous response shown in the clinical trial. This may be a limitation, but animals such as rabbits and guinea pigs are commonly used in experiments assessing the antigenicity of a new type of insulin prior to a clinical trial, and their results are considered to be relevant. Except for the silicone group control, we used insulin without any adjuvant in order to approximate clinical conditions as closely as possible. This immunization protocol was chosen since it effectively activated humoral IgG AIA in mice [28].

Since some authors had also used Wistar rats in immunization studies [27], we chose rats for practical reasons. The silicone control group confirmed that silicone might boost the antigenic response against insulin and confirmed that a significant antigenic response could be observed in this rat population.

Conclusion

The rat study shows that IP administered Hoechst 21PH insulin is less antigenic when administered directly from a vial than when it is administered after being stored in an implantable device during 6 weeks. It excludes the Genapol° or the IP route of administration as responsible factors for the high antigenicity of IP insulin infusion using implantable devices. With the necessity of remaining careful before extrapolating to humans, the hypothesis that the modifications due to the insulin storage in such conditions may be responsible for this increased antigenicity could be compatible with clinical data. Fibrillar aggregates could be good candidates since they are known to increase insulin antigenicity and have been found in significant concentration in the pumps. Further studies using calibrated aggregates concentrations in the same insulin are needed.

Acknowledgements

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Abbreviations

IP: intraperitoneal
AIA: Anti Insulin Antibody

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